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Amino-Acid Substitution in α-Spectrin Commonly Coinherited With Nondominant Hereditary Spherocytosis

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Nondominant hereditary spherocytosis (ndHS) is a disorder characterized in some patients by severe hemolytic anemia and marked deficiency of erythrocyte spectrin. This report describes the identification of a variant spectrin chain, α-spectrin Bughill or α^{BH}, that is associated with this disorder in a number of patients. Tryptic maps of spectrin from affected individuals revealed an acidic shift in isoelectric point of the αII domain peptides at 46 kD and 35 kD. A point mutation at codon 970 of the α-spectrin gene (GCT→GAT), that changes the encoded amino acid from an alanine to an aspartic acid, was identified in genomic DNA of affected patients. The α^{BH} variant was present in 8 patients with ndHS from five different kindreds but was absent in 4 patients from two other kindreds. The 8 ndHS patients with the α^{BH} variant appeared to be homozygous for the α^{BH} variant by analysis of peptide maps of limited tryptic digests of erythrocyte spectrin. However, following genomic DNA analysis, only 2 of these patients were true homozygotes, whereas 6 were found to be doubly heterozygous for the α^{BH} allele and a second, presumably abnormal, α-spectrin gene. These results suggest that, in these 6 patients, the second α-spectrin allele is in fact associated with one or more genetic defect(s), causing decreased accumulation of α-spectrin. The pattern of transmission of the α^{BH} allele in certain families suggests that the α^{BH} amino-acid substitution is not itself responsible for ndHS but is more likely a polymorphic variant that, in some but not all cases, is in linkage disequilibrium with another uncharacterized α-spectrin gene defect that itself is a cause of ndHS. Am. J. Hematol. 54:233–241, 1997

Key words: hereditary spherocytosis; α-spectrin gene; polymorphism, mutation; recessive disorder

INTRODUCTION

Hereditary spherocytosis (HS) is a common cause of inherited hemolytic anemia, affecting approximately 1 in 4,000–5,000 individuals in the United States and Europe [1–4]. HS is transmitted in an autosomal-dominant fashion in approximately 75% of cases. The remaining 25% of cases are believed to result either from spontaneous mutations or from a nondominant or recessively-inherited form of spherocytosis.

Patients with dominantly-inherited HS usually have a mild or moderate hemolytic anemia associated, in the majority of cases, with a modest deficiency of erythrocyte spectrin [5–8], the most abundant structural protein

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TABLE I. Hematological, Biochemical and Molecular Characteristics of Probands With ndHS

Family	Probands	Hct (%)	Retics (%)	Splenectomy	Spectrin content PAGE (%)	Spectrin content RIA (%)	α^{BH} peptides	α^{BH} allele	α^{LELY} allele	αI XbaI RFLP	αII XbaI RFLP
1	III-2	26.7	12	+	78	ND[a]	++	+/+	−/−	2/2	1/1
2	VII-3	39	1.2	+	68	59	++	+/+	−/−	2/2	1/1
	VIII-7	35	7	+	54	34	++	+/−	−/+	2/1	1/1
	VIII-8	32	9	+	52	31	++	+/−	−/+	2/1	1/1
3	V-I	51	2.9	+	74	52	0	−/−	−/−	2/2	1/1
	V-3	43	3.8	+	73	45	0	−/−	−/−	2/2	1/1
4	II-3	31	10	+	55	32	++	+/−	−/+	2/2	1/1
5	II-1	43	3.6	+	65	51	++	+/−	−/+	2/1	1/1
	II-2	40	3.3	+	71	57	++	+/−	−/+	2/1	1/1
6	III-3	31.6	9.6	+	43	33	0	−/−	−/−	1/2	1/2
	III-4	25.7	11.9	+	43	34	0	−/−	−/−	1/2	1/2
7	II-1	21.7	10	+	76	ND[a]	++	+/−	−/−	2/2	1/1

[a]ND, not determined.

of the erythrocyte membrane skeleton. In contrast, patients with nondominant HS (ndHS) often present with a severe or near-fatal form of hemolytic anemia in infancy or childhood, requiring frequent blood transfusions and early splenectomy [5–7]. Erythrocytes of these patients are characterized by a marked deficiency of spectrin, which can be as low as 30% of normal when measured by radioimmunoassay [6,7]. Both the severity of the disease and the clinical response to splenectomy in ndHS correlate with the magnitude of spectrin deficiency [7]. Parents of affected individuals are hematologically normal and have erythrocyte spectrin levels that are near the low end of the normal range, indicating little or no spectrin deficiency in heterozygous carriers of the disorder [5–7].

The cloning and characterization of many of the genes encoding erythrocyte membrane skeleton proteins have greatly facilitated the study and elucidation of specific molecular defects causing hereditary spherocytosis. A number of reports have been published that demonstrate the role of erythrocyte membrane skeleton proteins such as ankyrin, protein 4.2, the anion exchanger band 3, or β-spectrin in the pathogenesis of HS [1–4]. Dominant HS is thought to be caused primarily by mutations of the ankyrin or band 3 genes [1–4].

The underlying molecular defect(s) causing ndHS are less well-understood. Certain cases are associated with point mutations or other defects of the ankyrin gene, whereas other cases are found in association with missense mutations of the genes encoding band 3 or protein 4.2 [1–4]. Thus, ndHS appears to be a disorder characterized by considerable heterogeneity in its underlying molecular pathology.

We studied patients from four of the kindreds originally reported by Agre et al. [5–7], as well as three additional families with at least one individual affected by ndHS. This report describes the identification of a variant α-spectrin chain that is frequently coinherited with ndHS: two-dimensional peptide maps of limited tryptic digests of erythrocyte spectrin from affected individuals revealed an acidic shift in isoelectric point of the 46-kD and 35-kD αII domain peptides. This variant α-spectrin chain was originally designated as αIIa in our preliminary reports [9–11]. We have now identified a nucleotide base substitution in the α-spectrin gene that causes the amino-acid replacement responsible for the variant, and we have renamed it α-spectrin Bughill or α^{BH}, based on the place of residence in North Carolina of the original family described by Agre et al. [5,7]. Analysis of the inheritance patterns of the variant spectrin chain and its associated nucleotide base substitution in several kindreds with ndHS indicated that the base substitution and resulting amino-acid replacement are probably not themselves the cause of ndHS, but represent a polymorphism that is sometimes present in an α-spectrin gene that presumably carries a second mutation responsible for ndHS. A second α-spectrin allele, associated with very low levels of α-spectrin accumulation, is frequently inherited *in trans* to the variant α^{BH} allele in affected individuals with severe ndHS characterized by marked deficiency of erythrocyte spectrin. Some individuals with ndHS who lack the α^{BH} allele are presumably homozygous or doubly heterozygous for similar production-deficient α-spectrin alleles.

MATERIALS AND METHODS

Families With Nondominant Hereditary Spherocytosis

The hematologic findings in families 2–5 have been previously reported [5–7], and the family pedigrees presented (families A–D respectively) [7]. Typically, the affected individuals presented at birth with severe hemolytic anemia associated with spherocytes on peripheral blood smear [5–7]. Three additional families were also studied, and the hematologic findings of the affected probands in all seven families are listed in Table I. Families

2–5 were from North Carolina, whereas the other families (1, 6, and 7) were from other regions of the US without known familial ties to North Carolina.

Limited Tryptic Digestion of Spectrin

Erythrocyte ghosts were prepared from blood of affected individuals, as previously described [12]. Spectrin was extracted by incubating ghosts overnight at 4°C in low ionic strength buffer and stored in isotonic KCl. Spectrin extracts were dialyzed against 20 mmol/l Tris-HCl, pH 8.0, and 1 mmol/l β-mercaptoethanol for 20 hr at 4°C. Trypsin (TPCK [N-tosyl phenylalanine chloromethyl ketone]-treated, 206 U/mg; Cooper Biomedical, Malvern, PA) was added at an enzyme/substrate ratio of 1:20 wt/wt. The reaction time was 90 min at 2°C and was terminated by adding diisopropyl fluorophosphate (DFP) to a concentration of 1 mmol/l. The digests were lyophilized and stored at −80°C before electrophoresis.

Polyacrylamide Gel Electrophoresis (PAGE)

Spectrin tryptic digests (200 μg) were solubilized in 75 μl of a solution containing 9 mmol/l urea, 2% Triton X-100, 5% β-mercaptoethanol, and 2.4% ampholines (LKB Instruments, Inc., Rockville, MD), and focused on 3 × 125 mm polyacrylamide tube gels for isoelectric focusing for 5,700 volt hr [13]. After focusing, the gels were equilibrated for 10 min in 10% glycerol, 3% sodium dodecyl sulfate (SDS), 1 mmol/l EDTA, and 2% β-mercaptoethanol, and then frozen at −60°C before electrophoresis in the second dimension in 10–15% polyacrylamide gradient gels using buffers described by Laemmli [14].

Quantitation of Red Cell Spectrin Content

Erythrocyte membrane proteins were separated on 3.5–17% nonlinear gradient polyacrylamide gels using the buffer system of Fairbanks et al. [15], and stained with Coomassie blue. Spectrin/band 3 ratios were obtained either by the pyridine dye elution method [16], or by scanning of the stained gels on a Personal Densitometer laser scanner (Molecular Dynamics, Sunnyvale, CA) and calculation of the total volume of protein per band using Imagequant Software (Molecular Dynamics). In some cases, red cell spectrin content was also quantitated by radioimmunoassay (RIA), as previously described by Agre et al. [6].

Oligonucleotide Primers for Polymerase Chain Reaction

The nucleotide sequences of the primers used for amplification of the αII domain of the α-spectrin gene by the polymerase chain reaction (PCR) were as follows, with the indicated nucleotide (nt) positions of primers A–E in α-spectrin cDNA [17]: primer A, 5′-GTTCCAGCAGTACCTGGCTG-3′, nt 2886–2905, sense; primer B, 5′-CTGGCAGGCGTTTGCCTGAT-3′, nt 3065–3084, antisense; primer C, 5′-TTTGGAGACAGTATGAAAGC-3′, nt 3037–3056, sense; primer D, 5′-TTGATGGAACTGAGCAGCGT-3′, nt 3200–3219, antisense; primer E, 5′-GGACACTTTGCTGCAGAAGA-3′, nt 2774–2792, sense; and primer F, 5′-AGTGCGTGACTGACACCT-3′, intron 20, sense. Primer F corresponds to an intronic sequence 19 bases upstream of the 5′ intron/exon junction of exon 21 [18]. The intron between exons 19–20 is approximately 1.3 kb in length and that between exon 20–21, approximately 1.4 kb [18].

PCR Amplification of Genomic DNA and Nucleotide Sequencing

Genomic DNA from patients and their family members was prepared by standard procedures and amplified by PCR as described [19], using the following oligonucleotide primer pairs: A + B, B + E, or C + D. PCR products were subcloned into the pGEM7Z plasmid vector (Promega, Madison, WI), and the nucleotide sequence was determined by the dideoxynucleotide chain termination method of Sanger et al. [20], using the Sequenase enzyme kit (United States Biochemical Corp., Cleveland, OH). The sequencing primers used were the T7 and SP6 primers of the vector.

Allele-Specific Oligonucleotide Hybridization

Allele-specific oligonucleotide hybridization of PCR-amplified genomic DNA from patients and their family members was performed as described [21], except that the final wash condition was at 60°C for 10 min. The PCR primers used were primers D and F. The antisense oligonucleotide probes used for detection of the normal and mutant alleles had the following sequences: 5′-TCCACTGGTGCAGCCTGTTG-3′ (normal) and 5′-TCCACTGGTGCATCCTGTTG-3′ (mutant), respectively.

Restriction Enzyme Analysis of the α^{BH} Variant Allele

Genomic DNA was PCR-amplified as described above, using primers D and F, and then approximately 25% of the reaction mixture was digested with the restriction enzyme *Sfa*NI or *Fnu*4HI, in the appropriate buffer. The DNA fragments were then separated by electrophoresis in a 3.5% agarose gel, stained with ethidium bromide, and visualized under ultraviolet transillumination.

Analysis of Other Polymorphisms of the α-Spectrin Gene

We developed PCR-based assays [22,23] for the analysis of a number of previously published polymorphisms of the α-spectrin gene: the *Xba*I polymorphism of the αI domain [24], in intron 2; the *Xba*I polymorphism

of the αII domain [25], in intron 18; and the α^{LELY} polymorphism of the αIV–αV domain junction in exon 40 [26], that is associated with a lower-than-usual level of expression of the affected α-spectrin gene [27]. The chromosomal background or haplotype of the α^{BH} and other interacting α-spectrin alleles was thus determined in patients with ndHS.

RESULTS

Hematologic and Biochemical Characteristics of Nondominant Hereditary Spherocytosis

Table I lists the hematologic and biochemical features observed in 12 patients with ndHS characterized by moderate to severe RBC spectrin deficiency. As previously reported [5–7], the disorder usually presents with severe anemia requiring splenectomy at an early age. Following splenectomy, the degree of anemia is variable, but evidence of hemolysis usually persists, with elevated reticulocyte counts. Analysis of RBC spectrin content by RIA and by SDS-PAGE (spectrin/band 3 ratios) revealed decreased levels of spectrin ranging between 31–59% of normal (by RIA), or between 43–78% of normal (by SDS-PAGE). As previously noted [6], the spectrin/band 3 ratios determined by SDS-PAGE generally underestimate the true deficiency of RBC spectrin in this disorder, because of an associated reduction in band 3 content. In all seven families, the parents of the affected children were hematologically normal and their RBC had a normal spectrin content [5–7] (also, data not shown).

Two-dimensional fractionations of limit tryptic digests of RBC spectrin from family members revealed a frequent abnormality, as shown in Figure 1. Peptide maps from many asymptomatic parents revealed the presence of variant 46-kD and 35-kD peptides derived from the αII domain of spectrin (Fig. 1B). The variant peptides had an acidic shift in isoelectric point, compared to the normal αII domain peptides of 46 kD and 35 kD (Fig. 1A). The relative amounts of variant peptides were markedly increased in the affected ndHS children of such parents (Fig. 1C). The peptides were not directly quantitated, but a qualitative difference distinguished simple heterozygotes (parents) from ndHS patients with markedly increased levels of the variant peptides, i.e., absence of peptides 1 (46 kD) and 1′ (35 kD), as found in the sample of a true homozygote shown in Figure 1C. This pattern was consistently found in ndHS patients who inherited the variant α-spectrin gene. The α-spectrin variant, referred to as αIIa in prior preliminary reports [9,10], was seen in five of the seven families studied and in all 8 ndHS patients of these families. The 4 ndHS patients from the other two families lacked the variant peptides. In normal control individuals, presence of the variant peptides (heterozygous pattern) was observed in 6 of 147 subjects, or 4.1%.

A Base Substitution in the α-Spectrin Gene of an ndHS Patient

To determine the molecular basis of the αIIa variant, we analyzed the α-spectrin gene in a patient with nondominant hereditary spherocytosis (individual III-2, in family 1), who appeared to be homozygous for the condition by analysis of two-dimensional peptide maps of limited tryptic digests of erythrocyte spectrin (Fig. 1C). The region of the patient's genomic DNA containing exons 19–22, that encode the αII domain of α-spectrin, was amplified by PCR and subcloned into plasmid vectors. Nucleotide sequencing revealed a single base substitution (GCT to GAT) in codon 970 of the α-spectrin gene [17], that changed the encoded amino acid from an alanine to an aspartic acid (Fig. 2). This amino acid is at position 101 of the ninth 106-amino-acid repeat of α-spectrin [17], in a region that is not particularly well-conserved or homologous between different spectrin repeats; histidine is the most commonly encountered amino acid at this position in other spectrin repeats. This variant sequence in exon 21 was found in all six subclones that were sequenced, suggesting that the patient was in fact homozygous for the base substitution, in agreement with the results of protein analysis. No other recurrent base changes were found in the DNA sequence of the region 297 bases upstream and 102 bases downstream from the observed base substitution. The nature of the encoded amino-acid replacement (neutral to acidic amino acid) was consistent with the acidic shift in isoelectric point associated with the αIIa peptides, and the position of the base substitution at codon 970 was consistent with the predicted site of the amino-acid change, i.e., between the tryptic cleavage sites that generated the 35-kD and 30-kD fragments of the αII domain of spectrin. These results suggest that the observed base change is the likely cause of the αIIa variant peptides. The variant spectrin chain was named α-spectrin Bughill or α^{BH}, based on the place of residence in North Carolina of the original family described by Agre et al. [5,7].

Family Study by Allele-Specific Oligonucleotide Hybridization and Restriction Enzyme Digestion

To confirm that the α^{BH} variant is caused by the single base change detected in codon 970 of the α-spectrin gene of the proband, we screened other members of family 1 for presence of the base substitution. Genomic DNA from family members in the kindred was amplified by PCR and analyzed for the mutation by allele-specific oligonucleotide (ASO) hybridization (Fig. 3). The results showed that every family member with the α^{BH} variant peptides demonstrated by protein studies also carried the variant α-spectrin allele as demonstrated by molecular analysis (individuals I-2, I-3, II-1, II-2, and III-2 in Fig. 3). Family members who had only the normal αII peptide

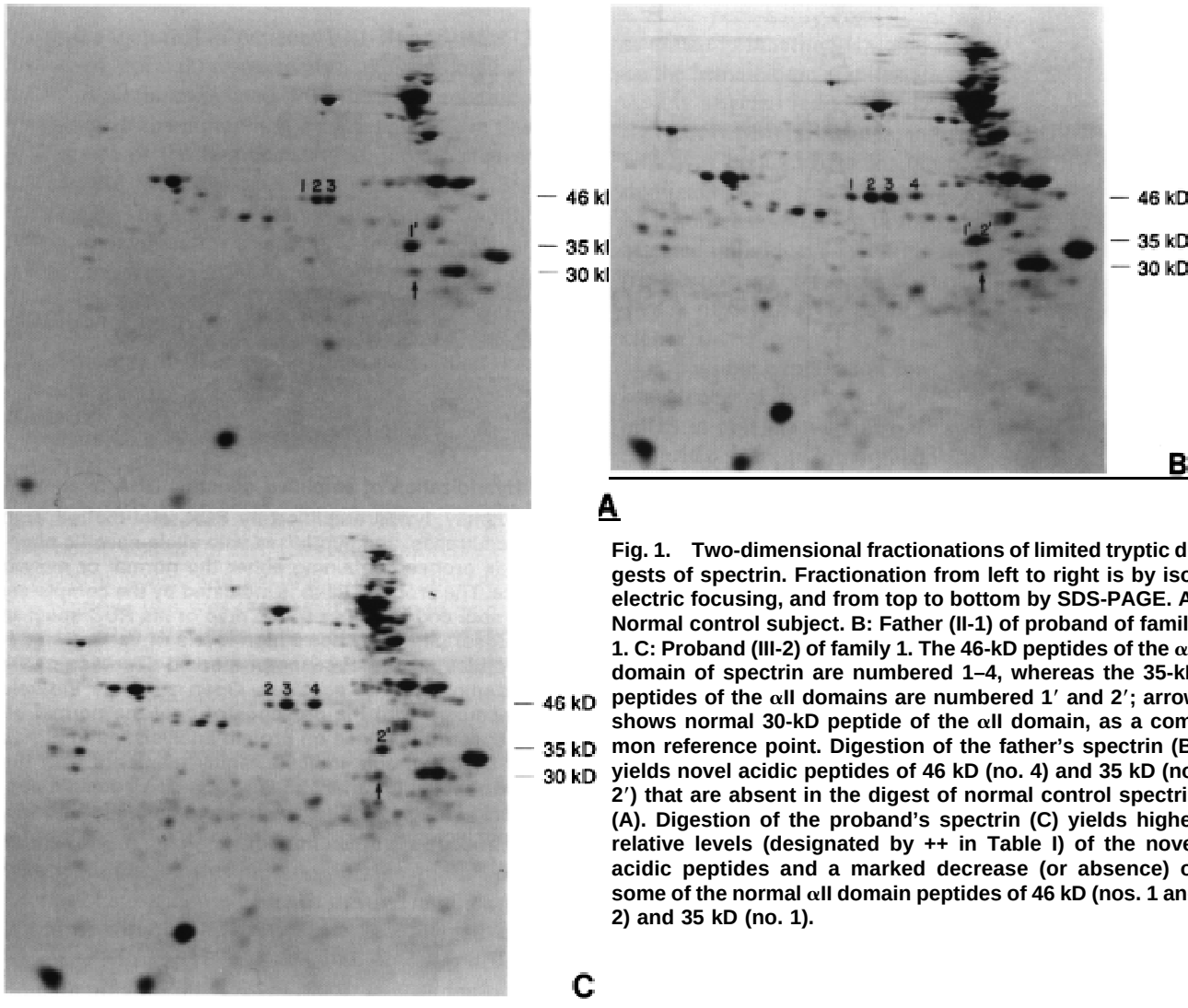

Fig. 1. Two-dimensional fractionations of limited tryptic digests of spectrin. Fractionation from left to right is by isoelectric focusing, and from top to bottom by SDS-PAGE. A: Normal control subject. B: Father (II-1) of proband of family 1. C: Proband (III-2) of family 1. The 46-kD peptides of the αII domain of spectrin are numbered 1–4, whereas the 35-kD peptides of the αII domains are numbered 1′ and 2′; arrow shows normal 30-kD peptide of the αII domain, as a common reference point. Digestion of the father's spectrin (B) yields novel acidic peptides of 46 kD (no. 4) and 35 kD (no. 2′) that are absent in the digest of normal control spectrin (A). Digestion of the proband's spectrin (C) yields higher relative levels (designated by ++ in Table I) of the novel acidic peptides and a marked decrease (or absence) of some of the normal αII domain peptides of 46 kD (nos. 1 and 2) and 35 kD (no. 1).

by protein analysis had only the normal allele (individuals I-1 and I-4). As expected, DNA of the proband (III-2), who appeared to be homozygous for the α^{BH} variant by protein analysis, contained only the variant α^{BH} allele. This strict coinheritance of the α^{BH} spectrin chain variant and the observed base substitution in exon 21 of the α-spectrin gene demonstrates that the base change is the cause of the α^{BH}-spectrin chain variant.

The base substitution in the α-spectrin gene associated with the α^{BH}-spectrin variant, GGCTGC to GGATGC, creates new restriction endonuclease cleavage sites for the enzymes *Fok*I (GGATG) and *Sfa*NI (GATGC), and abolishes cleavage sites for the enzymes *Bbv*I (GCTGC) and *Fnu*4HI (GCNGC). We made use of these findings to devise a rapid PCR-based assay to test for the presence of the base substitution. Genomic DNA was amplified by PCR using primers D and F, digested with *Sfa*NI, and fractionated by agarose gel electrophoresis: the normal allele yielded a single undigested fragment of 179 bp, whereas the mutant allele was cleaved by *Sfa*NI into two fragments, 138 bp and 41 bp in length (Fig. 4). Digestion of PCR products with the enzyme *Fnu*4HI gave appropriate results: bands of 134 bp and 45 bp for the normal allele, and a single undigested band of 179 bp for the mutant allele (data not shown). This assay was carried out using PCR-amplified DNA from members of family 1 (Fig. 4). The results confirmed the previously determined genotype of each individual, thus demonstrating the validity of the restriction enzyme-based assay.

Inheritance Patterns of the α^{BH} Allele in Patients With ndHS

We examined the pattern of inheritance of the α^{BH} allele in the various ndHS families in which the variant spectrin chain was found. In addition to the proband in family 1, 7 other ndHS patients (from four other families) had peptide maps with high relative levels of the variant peptides (as in Fig. 1C), in comparison to the lower levels seen in asymptomatic carriers (as in Fig. 1B). However,

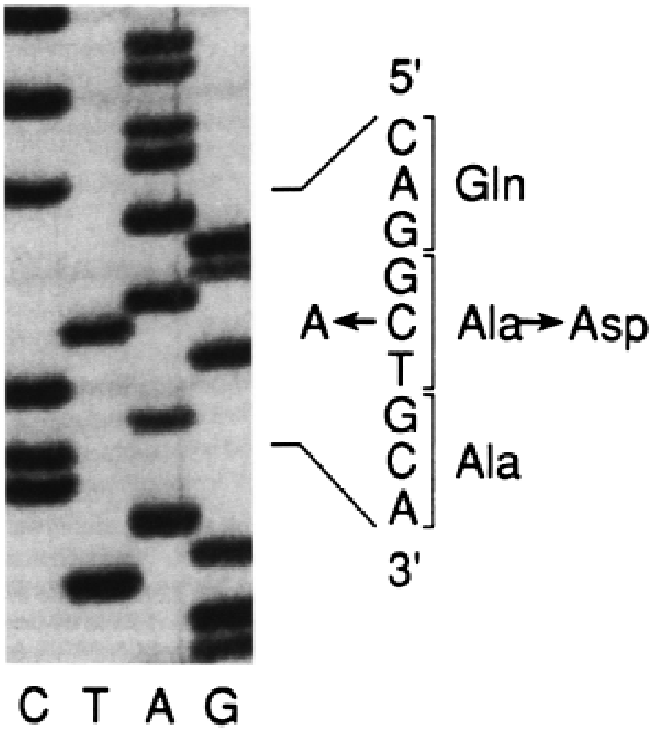

Fig. 2. Nucleotide sequence of exon 21 of the α-spectrin gene of the proband of family 1. Genomic DNA was amplified by PCR, and the product was subcloned in a plasmid vector for DNA sequencing. A base substitution was detected that changes a codon for alanine, GCT, to one for aspartic acid, GAT.

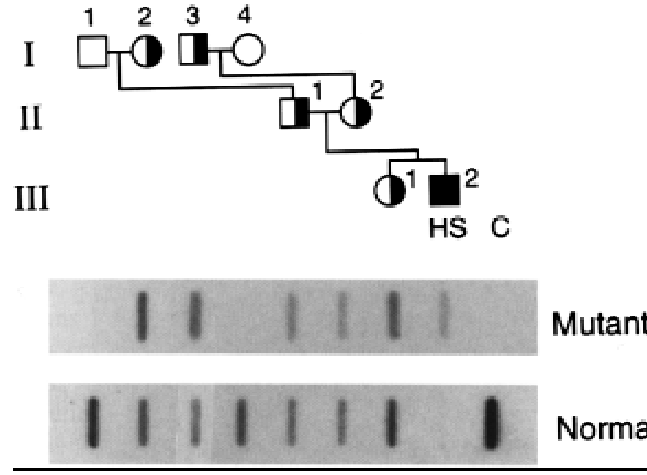

Fig. 3. Hybridization of amplified genomic DNA to allele-specific oligonucleotide probes. Genomic DNA from members of family 1 was amplified by PCR, slot-blotted onto nylon membranes, and hybridized with allele-specific oligonucleotide probes containing either the normal or mutant sequence. The proband (III-2) is indicated by the completely filled symbol and HS; the peptide map of his RBC spectrin tryptic digest (Fig. 1C) shows high levels of variant αII domain peptides and an absence or marked decrease of the corresponding normal peptides. Open symbols indicate family members with the presence of only the normal αII domain spectrin peptides on protein analysis, as in Figure 1A. Half-filled symbols indicate family members with the presence of both the variant and the normal αII domain peptides, as in Figure 1B. C: DNA from an unrelated normal control subject.

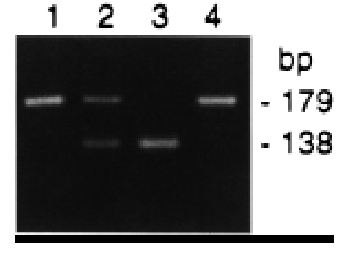

Fig. 4. Detection of α^{BH} allele by restriction endonuclease digestion of PCR-amplified genomic DNA. PCR products from members of family 1 (Fig. 3) were digested with *Sfa*NI, the fragments were separated by agarose gel electrophoresis, and the gel was stained with ethidium bromide. The base substitution in the α^{BH} allele created a new *Sfa*NI site, and fragments of 138 bp and 41 bp were generated after digestion of the PCR product with the enzyme. Lane 1, unaffected maternal grandmother (I-4); lane 2, heterozygous sister (III-1); lane 3, homozygous proband (III-2); lane 4, undigested control sample of proband.

6 of these 7 patients were found not to be homozygous for the α^{BH} allele, but to be doubly heterozygous for the α^{BH} allele and a second non-α^{BH} spectrin allele (Table I); we will refer to this second presumably mutant allele as allele 2. Therefore, the genotype of these patients is different from that of the proband in family 1 who was truly homozygous for the α^{BH} allele, despite the fact that the peptide maps of spectrin from all of the affected patients were strikingly similar to those shown in Figure 1C. In the cases where the genotype of the parents of the doubly heterozygous ndHS patients could be determined, one of the asymptomatic parents had low levels of α^{BH} variant peptides in limited tryptic digests of spectrin and was heterozygous for the α^{BH} allele, whereas the other parent lacked both the α^{BH} variant peptides and the α^{BH} allele (data not shown). These observations suggest that in the majority of the families studied, the disorder in the affected ndHS individuals arose from the coinheritance of the α^{BH} allele together with a second mutant α-spectrin allele, which is clinically silent in the heterozygous state and is transmitted to the ndHS patients by the parent without the α^{BH} allele. We detected only one other ndHS patient who was homozygous for the α^{BH} allele: patient VII-3 in family 2 (family A in Agre et al. [7]), who is distantly related to the original probands (VIII-7 and VIII-8) reported by Agre et al. [5]. These probands were doubly heterozygous for the α^{BH} allele and a second presumably mutant α-spectrin allele (allele 2), and were clinically more severely affected than patient VII-3. Interestingly, the erythrocyte membrane spectrin content of patient VII-3 (59% of normal) was less severely de-

creased than that of patients VIII-7 and VIII-8 (34% and 31% of normal, respectively), thus indicating that the α^{BH}-allele is associated with the accumulation of more α-spectrin chain than is α-spectrin allele 2 in this family.

In two of the families studied, the affected ndHS patients did not carry an α^{BH} allele (families 3 and 6 in Table I). The affected individuals in these families could be homozygous for a mutant α-spectrin allele similar to that proposed to exist in the doubly heterozygous ndHS patients with the α^{BH} allele, or else the ndHS in these patients with marked spectrin deficiency could be due to mutation(s) in the gene(s) encoding other membrane skeleton proteins.

Analysis of Polymorphisms in α-Spectrin Genes of ndHS Patients

We studied the chromosomal background of the α^{BH} and non-α^{BH} spectrin alleles in the various ndHS patients in order to search for characteristic haplotypes of mutant α-spectrin genes and to look for the presence of the α^{LELY} allele that was previously shown to be associated with a mild decrease in accumulation of α-spectrin chains [26,27]. The results of these studies are listed in Table I. In summary, the α^{BH} allele was found not to be associated with the α^{LELY} polymorphism *in cis* and, in all families studied, was associated with a single haplotype of αI and αII domain polymorphisms [24,25]. In three of the four families with doubly heterozygous ndHS patients (α^{BH}/α allele 2), the interacting α-spectrin allele 2 was positive for the presence of the α^{LELY} polymorphism. The α-spectrin genes in the two families (families 3 and 6) without the α^{BH} variant were negative for the presence of the α^{LELY} polymorphism. Two different αI domain polymorphisms were found in the non-α^{BH} spectrin alleles. Thus, there was heterogeneity in the chromosomal background (haplotype) of the non-α^{BH} spectrin genes of these ndHS patients.

DISCUSSION

We report on the identification of a nucleotide substitution in the α-spectrin gene that is associated with a variant spectrin, α-spectrin Bughill or α^{BH}, frequently found in patients having nondominant hereditary spherocytosis associated with moderate to marked deficiency of erythrocyte spectrin. In our studies, the α^{BH} variant was found in 4.1% of normal individuals from families without ndHS, whereas it was found in the majority, though not all, of the families having ndHS associated with moderate to marked deficiency of erythrocyte spectrin. These results suggest a close association between the α^{BH} allele and ndHS. In another study, Boivin et al. [28] found the α^{BH} (αIIa) allele in 7.6% of normal control subjects and in 12–14% of members from families with dominant HS. In these latter families, coinheritance of the α^{BH} allele was not necessarily concordant with the transmission of dominantly inherited HS, and had no apparent influence on the hematologic manifestations of the dominant HS in doubly affected individuals [28]. In particular, one family (family Mo) [28] had an individual with dominant HS who was homozygous for the α^{BH} allele; the cause of dominant HS in this family was subsequently shown to be a nonsense mutation in the band 3 gene [29], and the affected individuals, including the α^{BH} homozygote, had the phenotype of typical dominant HS with mild deficiency of erythrocyte band 3, rather than spectrin deficiency.

A possible explanation for these findings and for the prevalence of the variant α^{BH} allele in patients with ndHS is that the variant α^{BH} spectrin chain is itself a clinically silent polymorphism that, in some but not all cases, is in linkage disequilibrium with a second mutation of the α-spectrin gene that is a cause of ndHS. In other words, there must be two types of α^{BH} alleles: one allele without a second mutation, that is clinically silent even in the homozygous state, and another allele that carries a second mutation responsible for ndHS. Nondominant HS could result from homozygosity for the mutated α^{BH} allele or from double heterozygosity for the mutated α^{BH} allele and a second mutant α-spectrin gene that does not carry the α^{BH} polymorphism. A candidate mutation for the second defect linked to the subset of α^{BH} alleles causing ndHS was recently described by Wichterle et al. [30] and was named α-spectrin LEPRA (low-expression Prague). Recent studies of our patients for the presence of this second defect indicate that the α^{LEPRA} mutation is present in the α^{BH} genes of our ndHS families [31].

The elucidation of the molecular basis of the α^{BH} variant allowed us to use the mutation as a marker to determine the genotypes of affected individuals and to arrive at one important conclusion, namely that the second interacting α-spectrin gene (allele 2) is associated with markedly decreased levels of α-spectrin chain accumulation. As mentioned above, 2 of the ndHS patients examined were true homozygotes for the variant α^{BH} allele. Genetic analysis revealed that the 6 other patients were doubly heterozygous for the variant α^{BH} allele and a non-α^{BH} allele. In these 6 patients, analysis of peptide maps revealed a pattern similar to that observed in the α^{BH} homozygotes with predominance of the α^{BH} variant peptides, and with markedly decreased amounts of the corresponding non-α^{BH} peptides. This apparent discrepancy between the results of DNA and protein analysis raises the possibility that the gene product from the non-α^{BH}-spectrin allele (allele 2) of these 6 ndHS patients is, in fact, not assembled into the erythrocyte membrane skeleton. This finding is the strongest evidence that a defect in the expression of the α-spectrin gene is associated with ndHS in these families. The reason for this

lack of accumulation of gene product from the second α-spectrin allele may be due to a number of possible mechanisms. The mutant allele may be transcriptionally defective, its transcript may be unstable, or its gene product may be unstable or defective in its ability to bind and become assembled with other components of the membrane skeleton. Studies of α-spectrin gene polymorphisms in these families demonstrate that the second (non-α^{BH}) allele is genetically heterogeneous in different families, thereby indicating the possibility of multiple different mutations with similar phenotypes. The presence of heterozygosity for one or more polymorphisms in most ndHS patients who are not homozygous for α^{BH} also indicates the absence of a total deletion of one α-spectrin gene in these patients.

A number of genetic disorders of the α-spectrin gene have been described that are associated with decreased synthesis or accumulation of α-spectrin chains. Some patients with the syndrome of hereditary pyropoikilocytosis (HPP) [1–4,32] are doubly heterozygous for two different types of mutant α-spectrin genes: an α-spectrin gene encoding a structural α-chain variant associated with hereditary elliptocytosis (HE), and a second gene associated with markedly decreased accumulation of α-spectrin chains and concomitant marked deficiency of α-spectrin mRNA [33,34]. Two infants with hydrops fetalis due to severe nonimmune hemolytic anemia were born in a family in which one parent had apparently typical dominant HS and the other parent was hematologically normal [35]. Peripheral blood BFU-E-derived erythroid precursor cells cultured in vitro from one of the infants displayed total absence of α-spectrin chain synthesis [35]. The *sph/sph* type of recessive HS in the mouse is also characterized by virtual absence of α-spectrin synthesis and α-spectrin mRNA [36]. Finally, a less severe defect in α-spectrin chain accumulation, presumably due to alternative splicing of sequences encoded by exon 46, is associated with a polymorphic α-chain variant derived from the α^{LELY} allele [26,27]. In all of the above syndromes, the heterozygous carrier of the ''production-deficient'' α-spectrin gene, and even homozygotes for the less severe α^{LELY} allele, are hematologically normal. This phenomenon is probably explained by the fact that normal erythroid precursor cells synthesize 2–3 times as many α-spectrin as β-spectrin chains [37,38]. Thus, the output equivalent to that from a single normal α-spectrin gene is sufficient to provide enough α-spectrin chains to assemble with all of the β-spectrin chains in the cell, resulting in the formation of a normal membrane skeleton. Severe defects of α-spectrin chain synthesis or accumulation are therefore recessive and must be inherited in the homozygous or doubly heterozygous state in order to cause clinically significant disorders of the red cell [1–4].

Because of the established association of α-spectrin chain deficiency with the α^{LELY} allele, we tested the DNA from members of the affected families for the presence of the α^{LELY} allele (Table I, and data not shown). We found that the α^{BH} allele, as well as the second α-spectrin alleles in the ndHS patients of families 3 and 6 that lack α^{BH}, did not carry the α^{LELY} polymorphism. However, in 5 of the 6 ndHS patients doubly heterozygous for α^{BH} and a second non-α^{BH} allele, the second α-spectrin allele carried the α^{LELY} polymorphism. Nevertheless, we do not believe that the α^{LELY} defect itself is responsible for the marked deficiency of spectrin accumulation in these patients, because the deficiency of α-spectrin chain accumulation usually associated with the α^{LELY} allele is much milder and estimated to be the equivalent of a reduction of only approximately 50% of normal per α^{LELY} gene copy [26,27]. Therefore, a second mutation causing a more severe defect of α-spectrin chain production or accumulation may have occurred in the α^{LELY} allele inherited by the ndHS patients in families 2, 4, and 5. It is interesting to note that the degree of spectrin deficiency is generally less severe in the α^{BH} homozygotes than in the ndHS patients doubly heterozygous for α^{BH} and a second defective allele, as well as in the ndHS patients lacking α^{BH} altogether.

The study of α-spectrin gene polymorphisms in patients with ndHS and moderate to marked spectrin deficiency demonstrates the presence of multiple, different, associated α-spectrin gene haplotypes and suggests the likelihood of heterogeneity in the molecular basis of defective α-spectrin chain accumulation in these patients. Determination of the precise nature of these molecular defects will require the use of various screening techniques, such as those used in the study of other diseases, such as cystic fibrosis and Duchenne's muscular dystrophy, to scan the 52 exons of the α-spectrin gene for the presence of mutations.

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